

LETTER TO THE EDITOR.**Working title: Evaluating STZ-induced impaired wound healing in rats**

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To the editor,

A vast array of *in vivo* experimental models are utilised within the wound healing field. There remains little agreement as to the optimal *in vivo* experimental approach to mimic human chronic wounds (Wilhelm et al., 2017). Moreover, animal models of impaired healing have performed particularly poorly at translating drug based therapies to the clinic over many years, leading many to question their effectiveness (Gordillo et al., 2013). We have previously shown that the wound type (i.e. incision or excision) and subsequent analysis method will determine the sensitivity, and hence the likelihood of a statistically significant difference being correctly identified using a specific healing model (Ansell et al., 2014). When considered alongside our demonstration that rodent hair cycle can significantly alter the speed of repair (Ansell et al., 2011), careful experimental design of *in vivo* wound models becomes critical for maximising the probability of achieving statistical significance. Indeed the paucity of new drug based therapies emerging for the treatment of chronic wounds may be, at least in part, due to sub-optimal preclinical models.

Diabetes mellitus (DM) is a major cause of human chronic wounds (Eming et al., 2014). Several rodent models of DM are available (Ansell et al., 2012, Boyko et al., 2017, Davidson, 1998), however, the Streptozotocin (STZ)-induced DM model is almost exclusively used to model type 1 DM (Goodson and Hung, 1977). Like other wound models there is an inherent lack of consistency between published STZ-DM studies with variation in animal gender, wound size and wound type, but also in the length of time between DM-induction and subsequent injury (Table 1). We predict this latter variable to be crucial given that numerous effects of hyperglycaemia can take many weeks to manifest, such as advanced glycation end-product accumulation (Chen et al., 2009), or structural features of neuropathy (Biessels et al., 2014). To our knowledge, a rigorous assessment of the degree of healing impairment in the STZ model linked to time post induction has not been published.

To begin to fill this knowledge gap, we first examined wound healing following STZ-induced DM in male Wistar rats as this gender and strain combination is frequently used (Table 1). We compared healing in rats at 3- (n=6 rats) or 6-weeks (n=4 rats) post-DM induction to non-diabetic (n=6 rats) sham control rats (6mm punch biopsy wound harvested at 5 days post-wounding; see supplemental methods for full details), to assess the influence of time post-induction on healing outcome.

We collected wound photographs at day 5 (Figure 1a), which when assessed revealed a significant delay in healing (larger wound surface area) versus control only in 6-week post-DM induction animals (Figure 1b). To confirm this observation we profiled standardised histological wound parameters from tissue sections from the centre of each wound (Figure 1c). We found no statistically significant difference in histological wound width (Figure 1d)

or the area of wound granulation tissue (Figure 1e). Taken together, these data suggest that planimetry is a more reliable measure of overall healing delay in the STZ-DM model. Histological analysis, however, has merits with the parameter of re-epithelialisation demonstrating a statistically significant delay reduction (i.e. delayed wound closure) following 6 weeks of DM (Figure 1f). Again, there was only a trend towards delayed re-epithelialisation in rats 3 weeks post-DM induction. Thus, re-epithelialisation appears to provide a sensitive histological readout for the onset of impaired healing with DM.

The inability to statistically demonstrate any aspect of delayed healing at 3 weeks post-STZ-DM induction suggests that this (or any earlier) time point is insufficient, despite being used frequently in the literature (Table 1). We do however note a trend towards reduced re-epithelialisation at 3 weeks, which might become statistically detectable with increased sample sizes (Table S1). Many studies do not confirm whether significantly delayed repair exists with their chosen impaired healing model and sample size (Table 1). Furthermore, almost one third of studies do not indicate the DM induction timeframe used.

That only some wound parameters are demonstrably altered at 6 weeks post-STZ-DM induction suggests that the rate at which individual repair processes become impaired following loss of blood glucose control differs. To further explore this point we assessed changes in inflammation, collagen deposition and angiogenesis, with time-post STZ-DM induction. We find a strong increase in the number of wound macrophages in the 6 week STZ-DM group (Figure 1g), with no detectable difference in collagen deposition or angiogenesis (Figure 1h, 1i). While these data suggest that impairment of angiogenesis and collagen deposition take longer than 6 weeks post-STZ-DM, we cannot exclude our single analysis timepoint (day 5) providing a poor readout for these later phases of repair.

Pain withdrawal time in DM rats only declines 4-6 weeks post STZ administration (Kambiz et al., 2015), so few wounding studies will examine neuropathic healing. It would be interesting to assess chronic diabetes effects on healing, though this would necessitate use of insulin pellets, which will impact on the rate of healing (Goodson and Hung, 1977), and would preclude direct comparison to our earlier timepoints.

Our assessment of the literature revealed a strong preference for using male animals (Table 1), though the rationale for this remains unclear. To assess gender-specific effects on the STZ-DM model we conducted a second experiment comparing 6 weeks post-STZ-DM (n=9) versus non-diabetic (n=8) groups of female rats. Our macroscopic assessment reveals a significant delay after 6 weeks of diabetes, although the magnitude of difference was smaller than in males (Figure S1A). We could find no delay to repair by any histological measure in female rats (Figure S1B-D). Re-epithelialisation data were surprising given the pronounced delay observed in males (Compare Figure S1d to Figure 1f). Our data indicate that DM impaired healing is less pronounced in female rats. The underlying cause of this gender dichotomy remains unclear, though it may be related to the effects of sex steroid hormones (Ashcroft et al., 1997, Gilliver et al., 2009).

Collectively, these data indicate that numerous published studies have been performed using diabetic animals that display hyperglycaemia, but have not yet established a delayed healing phenotype, rendering the published observations invalid. While some may argue that in longer term post-wound studies rats will develop an impaired healing phenotype over the experimental window, early work showed that the initial wound response is critical for overall healing outcome (Seifter et al., 1981). Our study employed a small wound to correlate healing efficiency with a point in time, though this will not be the optimal approach for all

research questions. Our data highlight the importance of clear experimental design based on carefully validated wound models, in order to ensure that any treatment (e.g. drug) effect can be demonstrated, while reducing the overall requirements for animal use. Finally, our literature searches over the course of this study highlight an urgent need to improve the detail in reported experimental methodology, to include the age/weight, sex, strain and in the case of the STZ model the time post-STZ-DM induction, to allow the work to be properly compared to existing literature.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Profiling development of impaired wound healing following STZ induced diabetes. Wounds in male animals following 3 or 6 weeks of diabetes, were compared to a non-diabetic (ND) control. Representative macroscopic images of wounds at day 5 (a), were used to assess the wound surface area (b). Histology was taken through the centre of the wounds (c), to quantify the percentage of re-epithelialisation (d), wound width (e) and area of granulation tissue (f). Immunohistochemistry was used to assess for inflammation (Cd68; g), collagen deposition (Massons trichrome; h) and angiogenesis (Vwf; i). Bars expressed as mean \pm SEM, n=4 animals (6 week group) and 6 animals (ND and 3 week groups). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bar = 5mm (a) and 1mm (c).

Table 1. Publications in 2016 utilising a Streptozotocin induced diabetes, rat wound healing model*

	Group size	Strain	sex	weight/age	wound model	STZ induction	Impaired healing vs non-diabetic?	Reference
1	n=6	Wistar	Not stated	250-300g	1cm x 1cm circular excision	Not stated	Yes	Int J Biol Macromol. 2016 Aug 2;92:1162-1168
2	n=5-6	Sprague Dawley	Male	7wk	2cm diameter circular excision	up to 4 weeks	Yes	PLoS One. 2016 Jul 12;11(7):e0158647.
3	Not stated	Sprague Dawley	Not stated	250-300g	1.5cm x 1.5cm square excision	Not stated	No non-diabetic control arm	AAPS PharmSciTech. Epub 2016 Jun 29.
4	n=8	Wistar	Not stated	~200g (5wk)	12mm diameter circular excision	10 days	Not assessed	Life Sci. 2016 May 1;152:67-75.
5	n=3	Sprague Dawley	Male	300-325g	6mm diameter circular excision	3 weeks	Yes	J Diabetes Res. 2016;2016:5782904.
6	n=6	Wistar	Male	150-250g	6mm diameter circular excision	Not stated	Yes	Drug Des Devel Ther. 2016 May 24;10:1715-30
7	n=8	Sprague Dawley	Male	Not stated	1cm diameter circular excision	3 Days	No non-diabetic control arm	J Ethnopharmacol. 2016 Aug 2;189:277-89.
8	n=8	Wistar	Male	300-340g	6mm diameter circular excision	21 days	No non-diabetic control arm	Mol Cell Biochem. 2016 Jun;417(1-2):119-33.
9	n=6	Wistar	Not stated	150-200g	6mm diameter circular excision	Not stated	No non-diabetic control arm	Pharm Biol. 2016 May 14:1-5.
10	n=12	Wistar	Not stated	4 month	6cm x 5cm rectangular excision	4 weeks	Yes	Cell Transplant. 2016;25(1):71-81.
11	n=6	Lewis	Not stated	180-220g (6-9wks)	1.5cm diameter circular excision, splinted	4 weeks	No non-diabetic control arm	J Diabetes Complications. 2016 Sep 14. pii: S10
12	n=4-9	Wistar	Male	Not stated	Circular wound area 10mm ²	15 days	Yes	PLoS One. 2016 Oct 20;11(10):e0165115.
13	n=6	Sprague Dawley	Not Stated	6 wk	8mm diameter circular excision	Not stated	Not assessed	Adv Healthc Mater. Epub 2016 Nov 2.
14	n=20	Sprague Dawley	Male	300-400g	2cm x 2cm square wound	7 days	No non-diabetic control arm	Arch Dermatol Res. 2016 Jan;308(1):21-9.
15	n=5	Wistar	Male	180-22g	2cm x 2cm square wound	14 days	No non-diabetic control arm	Int Immunopharmacol. 2016 Jan;30:137-49.
16	n=8	Sprague Dawley	Male	300-400	2cm x 2cm square wound	16 days	Not assessed	Peptides. 2016 Apr;78:1-10.
17	n=5	Not stated	Male	250 +/- 50g	2cm diameter circular excision	Not stated	Yes	Sci Rep. 2016 Feb 23;6:19144.
18	n=10	Sprague Dawley	Male	250-300g	15mm diameter circular excision	14 days	No non-diabetic control arm	Int J Biol Sci. 2016 Nov 25;12(12):1472-1487.
19	n=6	Sprague Dawley	Mixed	250-350g	Circular wound area ~500mm ²	4 days	No non-diabetic control arm	J Interact Ethnopharmacol. 2016 Sep 25;5(4):43
20	n=12	Wistar	Male	200 +/-20g	6mm diameter circular excision	1 month	Yes	J Sci Food Agric. 2016 May;96(7):2337-43.
21	n=24	Not stated	Not stated	300-310g	6mm diameter circular excision	Not stated	No non-diabetic control arm	Iran J Pharm Res. 2016 Spring;15(2):583-9.
22	n=15	Wistar	Male	200-220g	2cm diameter circular excision	7 days	Not assessed	BMC Complement Altern Med. 2016 Oct 6;16(1)
23	n=8	Sprague Dawley	Male	210-240g	6mm diameter circular excision	3 days	Not assessed	J Ayurveda Integr Med. 2016 Oct - Dec;7(4):198
24	n=8	Wistar	Female	Not stated	6mm diameter circular excision	Not stated	No non-diabetic control arm	Arch Med Sci. 2016 Dec 1;12(6):1370-1376.
25	n=6-8	Sprague Dawley	Male	200-300g (6-8wks)	2cm diameter circular excision	Not stated	Yes	J Surg Res. 2017 Feb;208:93-103.
26	n=6	Sprague Dawley	Female	150-200g (4-6wks)	5mm circular diameter excision to hindfoot	4 weeks	No non-diabetic control arm	Stem Cell Res Ther. 2016 Oct 22;7(1):155.
27	n=12	Sprague Dawley	Mixed	200-250g (3 months)	1.8cm diameter wound	3 weeks	No non-diabetic control arm	Neural Regen Res. 2016 Mar;11(3):493-501.
28	n=7-10	Sprague Dawley	Male	290+/-10g	10mm diameter circular excision	4 weeks	Yes	J Diabetes Res. 2016;2016:5639129.

*42 pubmed listed publications were identified from 2016 using the search terms "streptozotocin skin wound healing rat". Only studies incorporating an *in vivo* excisional injury model were included for comparison. Papers that were not reported

